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Cytotoxic Activity of Benzothiepins against Human Oral Tumor Cell Lines

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Abstract. A total of 11 newly synthesized benzothiepins and structurally-related compounds were investigated for cytotoxic activity against both normal and tumor cells. All these compounds showed higher cytotoxic activity against three human oral tumor cell lines (HSC-2, HSC-3, HSG) than against normal human gingival fibroblast (HGF), suggesting tumor-specific cytotoxic action. In general, 3,4-dihydro-1-benzothiepin-5(2H)-ones ***[1-6]*** *showed higher cytotoxic activity than 2,3-dihydro-1-benzothiepins* ***[7-11]****. Compounds* ***4*** *(4-bromo-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5(2H)-one),* ***5*** *(4-bromo-3,4-dihydro-2-(2-oxopropyl)-1-benzothiepin-5(2H)-one) and* ***6*** *(4-bromo-3,4-dihydro-2-[1-(methoxycarbonyl)-1-methylethyl]-1-benzothiepin-5(2H)-one), showed higher cytotoxic activity than compounds* ***1, 2 and 3****, respectively, which had Cl instead of Br at C-4 position. Agarose gel electrophoresis demonstrated that these compounds induced large DNA fragments in oral tumor cells, whereas they produced smear pattern of smaller DNA fragments in human promyelocytic leukemia cells HL-60. These data suggest the medicinal efficacy of benzothiepins.*

Benzothiepin and benzothiepinone derivatives have been previously reported to exhibit the inhibitory activity of 5-lipoxygenase (**1**), a higher affinity for the muscarinic receptor (2) and inhibitory activity against platelet aggregation (3, 4). We have recently reported that newly synthesized 4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5(2*H*)-one [**1**] induced tumor-specific cytotoxicity and internucleosomal DNA fragmentation, a biochemical hallmark of apoptosis, in human leukemic cells (5). These findings prompted us to synthesize benzothiepin-related compounds, which might have higher cytotoxic activity against tumor cells.

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We investigated here the relative cytotoxic activity of newly-synthesized benzothiepins against human tumor cells [oral squamous cell carcinoma (HSC-2, HSC-3), salivary gland tumor (HSG) and promyelocytic leukemia (HL-60)] and normal cells [human gingival fibroblast (HGF)].

Materials and Methods

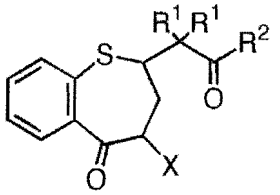
Materials. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM), RPMI1640 medium (Gibco BRL, Gland Island, NY, USA); fetal bovine serum (FBS)(JRH Biosci, Lenexa, KS, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind. Ltd., Osaka, Japan), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); RNase A, proteinase K (Boehringer Mannheim, Germany).

Synthesis of 3,4-dihydro-1-benzothiepin-5(2H)-ones (6) ***[1-6]*** *(general procedure A).* To a stirred solution of 7a-halo-1a,7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (7) (0.5 mmol) and silyl enolate (1.0 mmol) in acetonitril (MeCN) (4 mL), a solution of trimethylsilyl trifluoromethanesulfonate (TMSOTf) (33 mg, 0.15 mmol) in MeCN (0.5 mL) was added dropwise at 0°C under an argon atmosphere. After being stirred for 30 minutes, the reaction was quenched at the same temperature by adding saturated aqueous $NaHCO_3$ (2 mL). The mixture was stirred vigorously for 10 minutes and allowed to warm to room temperature. The mixture was extracted with CH_2Cl_2 (20 mL x 3), the combined organic layers were dried over Na_2SO_4, then the solvent was evaporated under reduced pressure. The residue was dissolved in tetrahydrofuran (THF)-1N HCl (2:1, 6 mL) and the solution was stirred for 1 hour at 0°C. The mixture was extracted with ether (20 mL x 3), the combined organic layers were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: hexane-AcOEt = 20: 1).

Synthesis of 4-chloro-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5(2H)-one [1]. According to the general procedure A, 7a-chloro-1a,7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (105 mg, 0.5 mmol) and 1-phenyl-1-(trimethylsilyloxy)ethylene (192 mg, 1.0 mmol) were treated with TMSOTf to give compound [**1**] (135 mg, 82%).

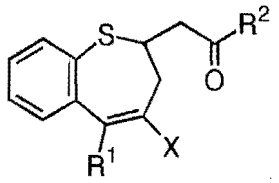
Synthesis of 4-chloro-3,4-dihydro-2-(2-oxopropyl)-1-benzothiepin-5(2H)-one [2]. According to the general procedure A, 7a-chloro-1a,7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (105 mg, 0.5 mmol) and 2-(trimethylsilyloxy)propene (130 mg, 1 mmol) were treated with TMSOTf to give compound [**2**] (115 mg, 86%).

3,4-dihydro-1-benzothiepin-5(2*H*)-one



1: R^1=H, R^2=Ph, X=Cl
2: R^1=H, R^2=Me, X=Cl
3: R^1=Me, R^2=OMe, X=Cl
4: R^1=H, R^2=Ph, X=Br
5: R^1=H, R^2=Me, X=Br
6: R^1=Me, R^2=OMe, X=Br

2,3-dihydro-1-benzothiepin



7: R^1=H, R^2=Ph, X=Cl
8: R^1=H, R^2=Ph, X=Br
9: R^1=H, R^2=OMe, X=Br
10: R^1=Me, R^2=Ph, X=Cl
11: R^1=Me, R^2=Me, X=Cl

Figure 1. *Structure of 3,4-dihydro-1-benzothiepin-5(2H)-ones [1-6] and 2,3-dihydro-1-benzothiepins [7-11].*

Synthesis of 4-chloro-3,4-dihydro-2-[1-(methoxycarbonyl)-1-methylethyl]-1-benzothiepin-5(2H)-one [3]. According to the general procedure A, 7a-chloro-1a, 7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (105 mg, 0.5 mmol) and 1-methoxy-2-methyl-1-(trimethysilyloxy)propene (174 mg, 1 mmol) were treated with TMSOTf to give compound [**3**] (143 mg, 92%).

Synthesis of 4-bromo-3,4-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin-5(2H)-one [4]. According to the general procedure A, 7a-bromo-1a,7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (127 mg, 0.5 mmol) and 1-phenyl-1-(trimethylsilyloxy)ethylene (192 mg, 1.0 mmol) were treated with TMSOTf to give compound [**4**] (160 mg, 86%).

Synthesis of 4-bromo-3,4-dihydro-2-(2-oxopropyl)-1-benzothiepin-5(2H)-one [5]. According to the general procedure A, 7a-bromo-1a,7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (127 mg, 0.5 mmol) and 2-(trimethylsilyloxy)propene (130 mg, 1 mmol) were treated with TMSOTf to give compound [**5**] (154 mg, 99%).

Synthesis of 4-bromo-3,4-dihydro-2-[1-(methoxycarbonyl)-1-methylethyl]-1-benzothiepin-5(2H)-one [6]. According to the general procedure A, 7a-bromo-1a,7a-dihydrobenzo[*b*]cyclopropa[*e*]thiopyran-7(1*H*)-one (127 mg, 0.5 mmol) and 1-methoxy-2-methyl-1-(trimethysilyloxy)propene (174 mg, 1 mmol) were treated with TMSOTf to give compound [**6**] (177 mg, 99%).

Synthesis of 2,3-dihydro-1-benzothiepins [7-11] (8) (general procedure B). To a stirred solution of 7a-halo-1,1a,7,7a-tetrahydrobenzo[*b*]cyclopropa[*e*]thiopyran-7-ol acetates (7, 9) (0.5 mmol) and silyl enolate (1.0 mmol) in MeCN (4 mL), a solution of TMSOTf (11 mg, 0.05 mmol) in MeCN (0.5 mL) was added dropwise at 0°C under argon atmosphere. After being stirred for 30 minutes, the reaction was quenched at the same temperature by adding saturated aqueous $NaHCO_3$ (2 mL). The mixture was stirred vigorously for 10 minutes and allowed to warm to room temperature. The mixture was extracted with CH_2Cl_2 (20 mL x 3), the combined organic layers were dried over Na_2SO_4, then the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (eluent: hexane-AcOEt = 50 : 1).

Synthesis of 4-chloro-2,3-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin [7]. According to the general procedure B, 7a-chloro-1,1a,7,7a-tetrahydrobenzo[*b*]cyclopropa[*e*]thiopyran-7-ol acetates (127 mg, 0.5 mmol) and 1-phenyl-1-(trimethylsilyloxy)ethylene (192 mg, 1.0 mmol) were treated with TMSOTf to give compound [**7**] (145 mg, 92%).

Synthesis of 4-bromo-2,3-dihydro-2-(2-oxo-2-phenylethyl)-1-benzothiepin [8]. According to the general procedure B, 7a-bromo-1,1a,7,7a-tetrahydrobenzo[*b*]cyclopropa[*e*]thiopyran-7-ol acetates (150 mg, 0.5 mmol) and 1-phenyl-1-(trimethylsilyloxy)ethylene (192 mg, 1.0 mmol) were treated with TMSOTf to give compound [**8**] (162 mg, 90%).

Synthesis of 4-bromo-2,3-dihydro-2-(methoxycarbonylmethyl)-1-benzothiepin [9]. According to the general procedure B, 7a-bromo-1,1a,7,7a-tetrahydrobenzo[*b*]cyclopropa[*e*]thiopyran-7-ol acetates (150 mg, 0.5 mmol) and 1-(*tert*-butyldimethylsilyloxy)-1-methoxyethylene (188 mg, 1.0 mmol) were treated with TMSOTf to give compound [**9**] (117 mg, 75%).

Synthesis of 4-chloro-2,3-dihydro-5-methyl-2-(2-oxo-2-phenylethyl)-1-benzothiepin [10]. According to the general procedure B, 7a-chloro-7-methyl-1,1a,7,7a-tetrahydrobenzo[*b*]cyclopropa[*e*]thiopyran-7-ol acetates (134 mg, 0.5 mmol) and 1-phenyl-1-(trimethylsilyloxy)ethylene (192 mg, 1.0 mmol) were treated with TMSOTf to give compound [**10**] (161 mg, 98%).

Synthesis of 4-chloro-2,3-dihydro-5-methyl-2-(2-oxopropyl)-1-benzothiepin [11]. According to the general procedure B, 7a-chloro-7-methyl-1, 1a,7,7a-tetrahydrobenzo[*b*]cyclopropa[*e*]thiopyran-7-ol acetates (134 mg, 0.5 mmol) and 2-(trimethylsilyloxy)propene (130 mg, 1 mmol) were treated with TMSOTf to give compound [**11**] (115 mg, 86%).

Cell culture. Human oral squamous cell carcinoma (HSC-2, HSC-3) and human salivary gland tumor (HSG) cells and human gingival fibroblast (HGF) (7-9th passage) were cultured in DMEM medium supplemented with 10 % heat-inactivated FBS in a humidified 5% CO_2 atmosphere. Human promyelocytic leukemia HL-60 cells were cultured in RPMI1640 medium supplemented with 10% FBS (5, 10, 11).

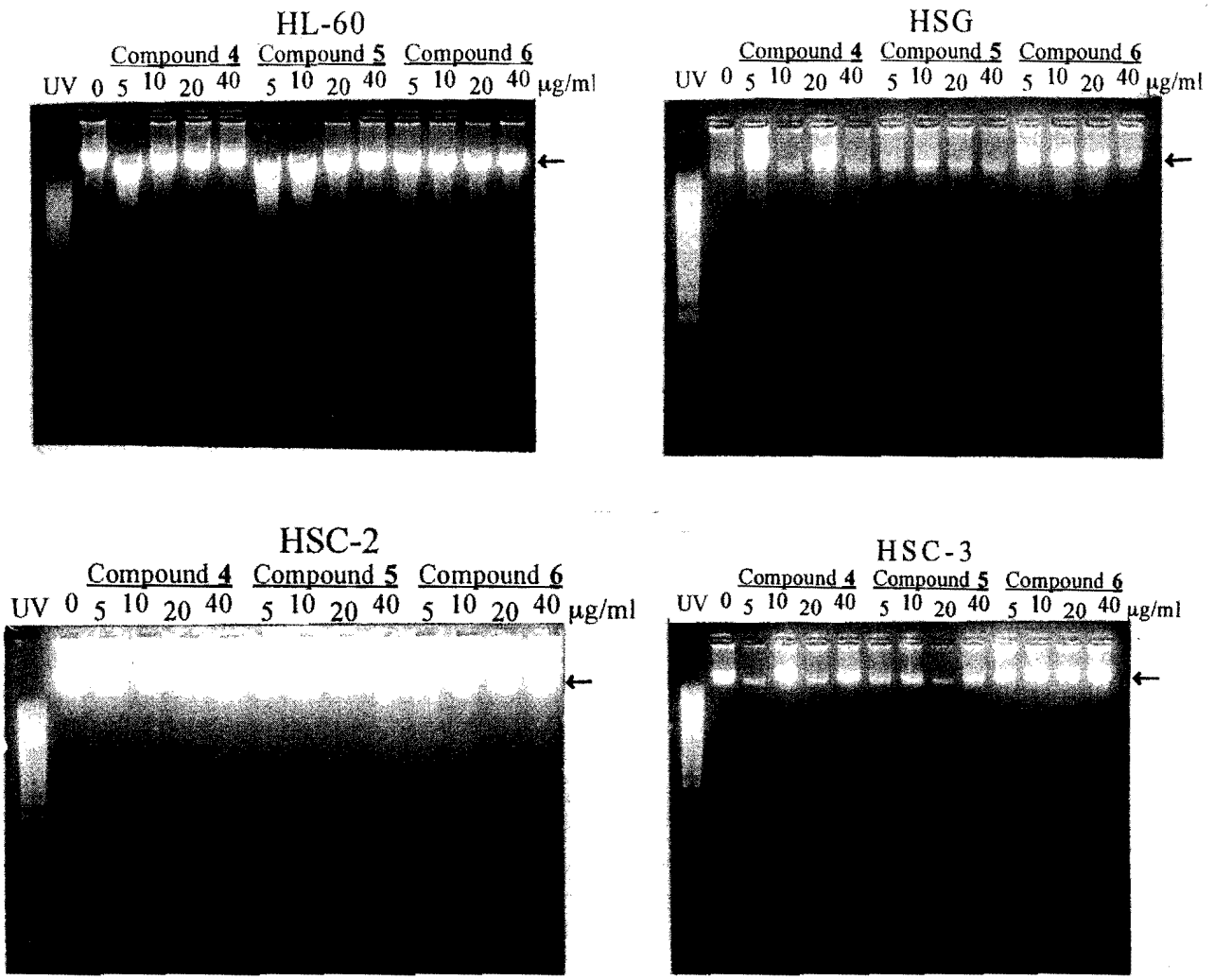


Figure 2. *Induction of DNA fragmentation by compounds* **4**, **5** *and* **6**. *Near confluent HL-60, HSG, HSC-2 and HSC-3 cells were incubated for 6 hours with 0 (control) (lane 2), 5 (lanes 3, 7, 11), 10 (lanes 4, 8, 12), 20 (lanes 5, 9, 13) or 20 (lanes 6, 10, 14) μg/mL of compound* **4** *(lane 3-6),* **5** *(lane 7-10) or* **6** *(lane 11-14). DNA was then extracted and subjected to 2% agarose gel electrophoresis. Lane 1 is the DNA from apoptotic HL-60 cells induced by UV irradiation (3).*

Assay for cytotoxic activity. Near confluent HSC-2, HSC-3, HSG and HGF cells grown in 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickenson) were incubated for 24 hours with various concentrations of samples. The cells were washed with phosphate-buffered saline (PBS) and incubated for 4 hours with fresh culture medium containing 0.2 mg/mL MTT. After removing the medium, the cells were lysed with 100 μl DMSO and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate with Labsystem Multiskan^{R} (Biochromatic) with Star/DOT Matrix Printer JL-10. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve (5, 10).

Assay for DNA fragmentation. Cells were pelleted, lysed and digested with RNase A and proteinase K. DNA was isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (5, 10). DNA from apoptotic HL-60 cells induced by UV irradiation (3) was run in parallel as a positive control.

Results and Discussion

A total of 11 benzothiepins and structurely-related compounds were newly synthesized (Figure 1). All these compounds showed higher cytotoxic activity against human squamous cell carcinoma (HSC-2, HSC-3) and human salivary gland tumor (HSG) than against human gingival fibroblast (HGF) (Table I). These data suggest the tumor-specific cytotoxic action of these compounds, although further systematic studies with many normal and tumor cell lines are necessary to confirm this point. In general, 3,4-dihydro-1-benzothiepin-5(2*H*)-ones [**1**-**6**] showed higher cytotoxic activity than 2,3-dihydro-1-benzothiepins [**7**-**11**]. The cytotoxic activity of compounds **4**, **5** and **6** which contained Br at C-4 position were slightly higher than that of compounds **1**, **2** and

Table I. *Cytotoxic activity and tumor specificity of benzothiepin-related compounds.*

Compound	Cytotoxic activity (CC_{50}: μg/mL)				Tumor specificity
	HSC-2 (A)	HSC-3	HSG	HGF (B)	B/A
3,4-dihydro-1-benzothiepin-5(2H)-one					
1	10	16	9	6	0.6
2	7	11	11	38	5.4
3	62	32	50	166	2.7
4	8	9	9	25	3.1
5	10	7	11	34	3.4
6	16	8	13	48	3.0
2,3-dihydro-1-benzothiepin					
7	440	432	>500	>500	>1.1
8	>500	>500	>500	>500	><1.0
9	>500	>500	>500	>500	><1.0
10	>500	>500	>500	>500	><1.0
11	51	65	73	238	4.7

Near confluent cells were incubated for 24 hours with various concentrations of each compound. The relative viable cell number was then determined by MTT method. Control A_{540} of HSC-2, HSC-3, HSG and HGF cells were 1.21, 0.96, 0.56 and 0.50, respectively. Each value represents the mean from 2 independent experiments which were done in duplicate.

3, which had Cl at the same position, respectively (Table I).

Agarose gel electrophoresis demonstrated that compounds **4**, **5** and **6** produced smear patterns of smaller DNA fragments in HL-60 cells (Figure 2). The optimum concentration for DNA fragmentation was very narrow and lower and higher concentrations did not produce smaller DNA fragments. On the other hand, compounds **4-6** produced only large DNA fragments (indicated by arrows), without producing smaller DNA fragments in oral tumor cell lines (HSC-2, HSC-3. HSG) (Figure 2). We have previously reported that vitamin K (12) and steroidal saponins (13, 14) failed to produce smaller DNA fragments in oral tumor cell lines. It remains to be investigated whether chromatin DNA of oral tumor cell lines is resistant to nuclease attack (15).

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